Treatment With OPN-305, a Humanized Anti–Toll-Like Receptor-2 Antibody, Reduces Myocardial Ischemia/Reperfusion Injury in Pigs

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Background—Toll-like receptor (TLR)-2 is an important mediator of innate immunity and ischemia/reperfusion-induced cardiac injury. We have previously shown that TLR2 inhibition reduces infarct size and improves cardiac function in mice. However, the therapeutic efficacy of a clinical grade humanized anti-TLR2 antibody, OPN-305, in a large-animal model remained to be addressed.

Methods and Results—Pigs (n=38) underwent 75 minutes ischemia followed by 24 hours of reperfusion. Saline or OPN-305 (12.5, 6.25, or 1.56 mg/kg) was infused intravenously 15 minutes before reperfusion. Cardiac function and geometry were assessed by echocardiography. Infarct size was calculated as the percentage of the area at risk and by serum Troponin-I levels. Flow cytometry analysis revealed specific binding of OPN-305 to porcine TLR2. In vivo, OPN-305 exhibited a secondary half-life of 8±2 days. Intravenous administration of OPN-305 before reperfusion significantly reduced infarct size (45% reduction, P=0.041) in a dose-dependent manner. In addition, pigs treated with OPN-305 exhibited a significant preservation of systolic performance in a dose-dependent fashion, whereas saline treatment completely diminished the contractile performance of the ischemic/reperfused myocardium.

Conclusions—OPN-305 significantly reduces infarct size and preserves cardiac function in pigs after ischemia/reperfusion injury. Hence, OPN-305 is a promising adjunctive therapeutic for patients with acute myocardial infarction. (Circ Cardiovasc Interv. 2012;5:279-287.)

Key Words: myocardial infarction ▪ reperfusion injury ▪ immune system ▪ Toll-like receptors

Blood flow restoration through the culprit coronary artery is necessary to save endangered myocardium after acute myocardial infarction (MI). It is well established that early reperfusion limits infarct size (IS) and improves clinical outcome of patients with acute MI.1 Nevertheless, current reperfusion therapy remains suboptimal because complications after MI are an increasing burden to society. Furthermore, additional cell death occurs during the reperfusion phase caused by detrimental inflammatory responses.2,3 Innate immune responses are major contributors to cell death through deleterious proinflammatory cytokine release and cell-to-cell interactions between leukocytes and cardiomyocytes. Previous experiments clearly show a “window of opportunity” for adjunctive therapeutic strategies targeting innate immune responses to increase myocardial viability and survival.4 Unfortunately, most immune modulating interventions that have proven to be effective in experimental studies failed in the clinical setting.5 For example, drug administration before the ischemic period and lack of large-animal testing are key reasons for the failure of successful translation in the clinic. To achieve clinical relevance, experimental compounds must be administered in the late ischemic period or during reperfusion, while large animals have greater physiological relevance to human compared with murine models of MI.6,7

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Ischemic injury triggers the activation of innate immunity, and this is augmented after reperfusion, resulting in larger IS and therefore hampering the true potential of reperfusion therapy.2 Adjunctive therapies inhibiting innate immunity have been shown to further limit IS after myocardial ische-

Received February 22, 2011; accepted January 12, 2012.

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Circ Cardiovasc Interv is available at http://circinterventions.ahajournals.org DOI: 10.1161/CIRCINTERVENTIONS.111.967596

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Myocardial ischemia/reperfusion (I/R). However, a completely abolished inflammatory response is detrimental for repair responses because inflammation is critical for proper wound healing.

For example, corticosteroid administration reduces IS but results in aneurysm formation and rupture of the myocardium after infarction.

Thus, targeted therapy is critical to reduce reperfusion injury and leave essential wound-healing mechanisms intact.

The discovery of Toll-like receptors (TLRs) has increased the therapeutic potential of innate immune modulating interventions. Originally thought of as pathogen recognition receptors for exogenous pathogens, TLRs have also been shown to recognize endogenous molecules released after tissue injury, also referred to as “danger signals.” These danger signals released after cell death are thought to engage with TLRs to induce inflammation. TLR2 expression on bone marrow–derived cells mediates I/R injury. Systemic administration of a monoclonal anti-TLR2 antibody reduces infarct size and enhances cardiac performance in mice after myocardial I/R injury. Many murine therapeutic successes fail in large animal models.

WHAT THE STUDY ADDS

For the first time, a clinical-grade humanized anti-TLR2 antibody (OPN-305) is used in a porcine model of myocardial infarction, which resembles human anatomy and physiology.

OPN-305 has a convenient short half-life of approximately 8 days.

OPN-305 reduces infarct size and enhances cardiac function in a porcine model of myocardial I/R injury.

OPN-305 is a good candidate to be tested as an adjunctive therapeutic for patients with myocardial infarction to limit I/R injury and reduce infarct size.

WHAT IS KNOWN

- Myocardial ischemia/reperfusion (I/R) triggers deleterious innate immune responses resulting in increased infarct size and decreased cardiac function.
- TLR2 receptors (TLRs) play a significant role in innate immunity.
- TLR2 expression on bone marrow–derived cells mediates I/R injury.
- Systemic administration of a monoclonal anti-TLR2 antibody reduces infarct size and enhances cardiac performance in mice after myocardial I/R injury.
- Many murine therapeutic successes fail in large animal models.

Administration of OPN-305 to Determine Half-Life

OPN-305 was administered intravenously to a separate group of 12 pigs (4 per group) at the following doses: 6.25 mg/kg, 12.5 mg/kg, and 25 mg/kg. The pigs were bled through an indwelling central venous catheter at the following time points: before administration; at 5 and 30 minutes; 1, 2, 4, 6, 12, and 24 hours; and 2, 3, 4, 7, 10, 14, 18, 22, 28, 35, and 42 days after OPN-305 injection. Plasma was derived from the samples, aliquoted, and frozen at −20°C. Determination of OPN305 in plasma was achieved using a quantitative ELISA-based approach. The lowest dose used in the efficacy study was not included in the pharmacokinetics study because it was thought that the ELISA would not be sensitive enough to detect antibody clearance. This does not affect the results of the pharmacokinetics study because the half-life is not affected by dose.

FACS Staining of Cells

Porcine peripheral blood mononuclear cells were purified with the use of Polymorphprep (Axis-Shield) as per manufacturer’s instructions. Cells were washed and resuspended in 1% BSA in PBS. Cells were counted and 1×10⁶ cells were used per tube. OPN-305 was used to stain porcine cells and a polyclonal preparation of human IgG4 (Sigma) was used as the isotype control staining because there was no monoclonal IgG4 isotype available. Binding was detected with PE-conjugated anti-human IgG4 (Southern Biotech). All incubations were carried out on ice for 25 minutes with 3× washing in 1% BSA between each antibody step. Cells were fixed with 1% paraformaldehyde and acquired with the use of a Dako Cyan before analysis using Summit version 4.3.
OPN-305 ELISA

In brief, the ELISA format uses His-tagged TLR2 (50 ng/mL) bound to nickel-coated plates. After blocking with 1% BSA/0.05% Tween 20 in PBS, a standard curve of OPN-305 and quality control samples were added to the plate. Bound OPN-305 was detected using biotinylated anti-human IgG4 (1/5000) followed by conjugation of streptavidin HRP (1/5000). The signal was visualized using TMB and the reaction was stopped using 1 mol/L sulfuric acid. The assay range in 10% porcine plasma was 1000–15,625 ng/mL.

For each assay run to pass, it must reach the following criteria: (1) At least 75% of the calibration points must be within ±20% of the nominal concentration (% recovery of the back-calculated values); duplicate measurements of individual calibration points must have a coefficient of variation of ≤20%. (2) Mean intrabatch precision is within ±20% coefficient of variation for the validation quality control samples. (3) Mean intrabatch accuracy for validation quality control samples must be within ±20% of the nominal concentration (% recovery of the back-calculated values). (4) At least 67% of the quality control samples should be within 20% of their respective nominal (theoretical) values: 33% of the quality control samples can be outside the ±20% nominal value. (5) During the pharmacokinetics analysis, samples were diluted to ensure that they fall in the range of the curve. In the instance, for example, where a sample when diluted 1/100 and 1/1000 falls on the standard curve, the reading that has the (1) lowest % coefficient of variation and (2) is falling on the linear part of the curve (not at the very top or very bottom of the curve) was taken as the concentration of the sample.

Premedication and Anesthesia

All animals were pretreated with amiodarone (400 mg/d; start 10 days before surgery), clopidogrel (75 mg/d; start 3 days before surgery), and acetylsalicylic acid (80 mg/d; start 10 days before surgery). To treat postsurgical pain, pigs were given fentanyl patches (25 μg/h; Duragesic Janssen-Cilag, Tilburg The Netherlands) 1 day before surgery.

Pigs were sedated with intramuscular injection with ketamine (10 mg/kg), midazolam (0.5 mg/kg), and atropine (0.04 mg/kg). Hereafter, sodium thiopental (5 mg/kg) was given intravenously, before intubation. Intravenous administration of amoxicillin/clavulanic acid (1000/100 mg) was given before surgery.

Before ventilation (tidal volume, 10 mL/kg; respiration rate, 12/min; oxygen/air mixture), a bolus injection of midazolam (0.5 mg/kg) and sufentanil citrate (6 μg/kg) was given intravenously. Metoprolol (5 mg) was administered before and after infarction. Amiodarone (300 mg) was given intravenously before incision.

Pigs were kept under anesthesia during the surgical procedure using intravenous infusions of midazolam (1 mg/kg per hour), sufentanil citrate (6 μg/kg per hour), and pancuronium bromide (0.1 mg/kg per hour).

Surgical Procedures

For the pharmacokinetic studies, percutaneous tunneled sterile silicone central venous catheters (right external jugular vein) were inserted and flushed daily with 250 IE heparin diluted in 5 mL saline. The tip of the catheter was placed 10 cm through the external jugular vein in the vena cava superior, while the syringe fitting end was placed dorsally in the neck for easy conscious blood drawing. Before drawing the first sample for analysis after OPN305 administration, the lines were flushed with saline to prevent contamination of the blood sample with OPN305 that may be present in the lines.

For the induction of infarctions, pigs were anesthetized and ECG, arterial blood pressure, and capnogram were continuously monitored. Core body temperature was kept constant at 37°C. A midsternotomy was performed to open the mediastinal cavity. The pericardium was opened, and a 2–0 Prolene suture was placed beneath the proximal left circumflex (LCx) coronary artery. The ligation was done right after the bifurcation of the left main coronary artery into the left anterior descending coronary artery and LCx and before any proximal side branches of the LCx. Heparin (15 000 IE) was given to prevent thrombus formation in the LCx during and after ischemia. Ischemia was induced by ligating the LCx between the suture and a polyethylene tube. Ischemia was confirmed by ECG. Reperfusion was initiated by releasing the ligature and removing the polyethylene tubing. A piece of the loosened suture was left in place to determine the ischemic area on termination. Thoracic drains were placed to remove excessive fluid and air from the thoracic cavity and the sternum was closed. Pigs were weaned from mechanical ventilation and returned to their pens.

Echocardiography

End-diastolic volume and end-systolic volume (EDV and ESV), ejection fraction (EF), wall thickness (WT), systolic wall thickening (SWT), and fractional shortening (FS) were determined using short- and long-axis transthoracic ultrasound image acquisition. Echocardiography was performed at baseline and 24 hours after reperfusion. Short- and long-axis images were obtained for EF calculation. WT, SWT, and FS were calculated at basal level on short-axis images. EF=100*(EDV−ESV)/EDV; SWT=100*(wall thickness ED−wall thickness ES)/wall thickness ED; FS=100*(ED diameter−ES diameter)/ED diameter.

Infarct Size

IS was assessed 24 hours after I/R injury and expressed as a percentage of the area at risk (AAR). The ratio AAR/LV is a measure for the extent of myocardial tissue that underwent ischemia and reperfusion (ie, endangered area; Figure 1). The ratio IS/AAR is an accurate measure to determine IS within the endangered myocardium and is the primary end point from which the efficacy of treatment is addressed.

To determine the AAR, the LCx was ligated again (at the level marked by the suture left in place) and 50 mL 4% Evans blue dye was injected via the apex in the left ventricular cavity. Hearts were rapidly explanted, rinsed in 0.9% saline, and mechanically sliced into 4–5 cross sections. Heart sections were weighed and incubated in 1% triphenyltetrazolium chloride (Sigma-Aldrich) at 37°C for 10 minutes. Viable tissue stains red and infarcted tissue appears white. Heart sections were digitally photographed (Canon EOS 400D) under a microscope (Carl Zeiss). IS, AAR, and total LV area were measured using ImageJ software (version 1.43) and corrected for the weight of the corresponding slice.

Exclusion Criteria

Operated pigs were excluded for all analysis, based on a small AAR (AAR/LV <20%) and failure to reperfuse the ischemic myocardium (IS/AAR >95%).

Statistical Analysis

All continuous data are expressed as mean±SEM. To correct for multiple comparisons, 1-way ANOVA with post hoc Dunnett t test was used to compare means between groups (saline treatment was set as control; ANOVA indicated significant differences in means across the groups). A Pearson correlation test was done to assess the accuracy of the IS using Troponin I levels compared in comparison with invasive infarct staining (Evans blue dye with TTC staining), 24 hours after reperfusion. All statistical analyses were performed with the use of SPSS 15.1.1 for Windows; P<0.05 was considered significant.

Results

Specificity and Half-Life Determination of OPN-305

As shown in Figure 2, the humanized anti-TLR2 antibody OPN-305 binds specifically to porcine TLR2. Hereafter, we characterized the pharmacokinetics of OPN-305 by intravenous administration at 3 different doses (n=4/group) in pigs: 25, 12.5, and 6.25 mg/kg. An OPN-305–specific ELISA was developed at Opsona Therapeutics Ltd in the presence of...
pooled pig plasma (10% final dilution) to determine the half-life of OPN-305. As shown in Figure 2, there is a dose-dependent Cmax, as expected, and all doses exhibit a half-life of 8 days.

**OPN-305 Treatment Before Reperfusion Reduces IS**

Having established the pharmacokinetics and binding capacity of OPN-305 to porcine cells, we investigated the therapeutic efficacy of inhibiting TLR2-mediated myocardial I/R injury in pigs.

Thirty-eight pigs underwent myocardial I/R injury. One pig (6.25 mg/kg treated) died after surgery as the result of postinfarct ventricular fibrillation.

Five animals met the exclusion criteria and were excluded from all analyses (3 pigs with 20% AAR; 2 pigs 95% infarction). Intravenous OPN-305 administration 15 minutes before reperfusion reduced IS by approximately 45%, in a dose-dependent manner (IS/AAR; Figure 3A). The extent of endangered myocardium during ischemia (AAR/LV) did not statistically differ between the groups (Figure 3B), indicating surgical and anatomic consistency. In addition, the extent of myocardial necrosis assessed by serum levels of Troponin I was also significantly reduced in OPN-305–treated animals in a dose-dependent fashion (Figure 3C). The IS assessment in peripheral blood appeared to be an accurate reflection of the actual extent of necrotic myocardium, since there was a strong correlation between Troponin I levels and IS determined by TTC staining (Pearson correlation, 0.593 at P = 0.001 level). Finally, we measured white blood cell (WBC) counts in peripheral blood 1 day after reperfusion because high WBC counts after infarction are associated with worse clinical outcome. Although there were no statistical differences between the groups, there was a clear trend to lower WBC count in the effective infarct reducing dose of 12.5 mg/kg OPN-305–treated animals (P = 0.186 compared with saline).

**OPN-305 Improves Systolic Function After Myocardial I/R Injury**

Transthoracic 2-dimensional echocardiographic analyses revealed no differences between the groups in cardiac function and geometry at baseline. One day after reperfusion, there was no difference in global EF between the groups (Figure 4A). Again, in a dose-dependent fashion, FS was higher in OPN-305–treated animals (Figure 4B). In line with decreased IS and improved FS, local systolic function (SWT) of the infarct area was significantly preserved in OPN-305–treated animals (Figure 4C). Whereas saline treatment resulted in complete loss of contraction of the infarct area (as shown by a negative SWT index), OPN-305 treatment significantly preserved systolic performance in a dose-dependent manner. There was no difference in the amount of reperfusion-induced edema in the infarcted myocardium after 24 hours (WT; Figure 4D).

**Discussion**

Current therapy for patients with acute MI is early reperfusion of the ischemic myocardium. Recent technical (eg, stents) and pharmacological (eg, glycoprotein IIaIIIb inhibitors) advances have resulted in a significant decline of
infarct-related deaths over the past decade. However, increased survival after acute MI also led to increased morbidity due to excessive tissue loss in the surviving patients. Fortunately, experimental and clinical studies have clearly demonstrated that adjunctive therapeutics to reperfusion offer the potential to further enhance myocardial viability after infarction. One of the potential therapeutic options is to reduce detrimental activation of innate immunity after myocardial I/R injury. TLRs have been shown to be a critical mediator of innate immunity as a first-line defense against pathogens as well as ischemia-induced cardiac injury.4,18,19 Injury-related endogenous activators, referred to as “danger signals,” released after cell death are thought to activate TLRs and initiate deleterious inflammatory responses.15–18 There is a great amount of evidence that shows the pivotal role of TLR2 in I/R injury of various organs.8 Previous studies have clearly demonstrated that TLR2 mediates I/R injury of the myocardium and endothelial cells.22,23 Recently, we have shown that myocardial I/R injury is mediated by expression of TLR2 on circulating cells. More importantly, OPN-301 (a mouse anti-TLR2 antibody) administration just before reperfusion reduced IS and improved cardiac function in mice.24 These data provided a rationale to assess the therapeutic efficacy of a humanized anti-TLR2 antibody, OPN-305, which is the humanized clinical candidate of parent antibody OPN-301. In the present study, we determined the therapeutic potential of TLR2 inhibition by using a porcine myocardial I/R model that is more physiologically relevant to the human situation compared with mice.

First, we confirmed that OPN-305 bound specifically to porcine TLR2. In vivo, OPN-305 exhibited a secondary half-life of approximately 8 days. The relatively short half-life reduces long-term inhibition of TLR2, thereby decreasing the risk for a potential unfavorable effects of chronic TLR2 inhibition.

Second, we assessed the therapeutic efficacy of OPN-305 by using a porcine myocardial I/R injury model. In line with our previous study,24 we observed a significant reduction in IS of approximately 45% in 12.5 mg/kg OPN-305–treated animals compared with saline-treated pigs. We also assessed the efficacy in 6.25 and 1.56 mg/kg OPN-305, demonstrating a dose-dependence curve. We expected to observe to some...
For this reason, we went 4-fold down from 6.25 to 1.56 mg/kg for our lowest dose. We also determined the extent of myocardial necrosis by measuring Troponin I levels in peripheral blood, drawn 1 day after reperfusion. Troponin I is a widely used diagnostic tool for acute MI but also correlates with the extent of irreversible myocardial injury in humans.29

In line with the invasive findings, IS assessed by serum
Troponin I levels was also reduced in OPN-305–treated animal in a dose-dependent manner. Next, we plotted the TTC-based IS of the pigs against the corresponding Troponin I levels (Figure 3D). There was a highly significant correlation between the invasive IS measurements and the Troponin I measurements ($r = 0.593; P = 0.001$), demonstrating that a simple biochemical tool such as Troponin I measurement is a reliable way to assess the efficacy of OPN-305 in a future clinical study. In addition, WBC counts may also be used to as a secondary parameter for efficacy or prognosis. However, in our study, this clearly appeared to be suboptimal because the surgical procedure (sternotomy) itself is a strong trigger for leukocytosis and is likely to have caused significant background noise. Therefore, in this surgical setting, it is almost impossible to observe the leukocytosis only caused by myocardial I/R injury.

Five animals were excluded for 2 reasons: AAR too small and failure to reperfuse the ischemic myocardium. AAR is one of the most important determinants of final IS: the greater the AAR, the larger the infarct within the left ventricle. The size of the AAR is determined by the extent of myocardium perfused by (1) the culprit coronary artery and (2) collateral flow. In our study, ischemia was induced by ligating the proximal LCx coronary artery. Previous experiments showed that coronary ligation at this site results in approximately 33% of endangered myocardium within the left ventricle.30 The relatively small AAR in 3 pigs is probably due to anatomic variation seen in outbred animals.7 Despite the coronary occlusion at the very proximal level, collateral flow may have caused sufficient perfusion behind the ligation. This notion is supported by the fact that 2 excluded pigs had no infarction at all, despite 75 minutes of coronary occlusion. The 3rd pig showed 100% infarction within the small AAR (6%), which resembles a nonperfused ischemia model. For this reason, we excluded 2 pigs with >95% infarction. In addition to AAR, the ischemic duration is another important determinant of final IS. We used 75 minutes of ischemia followed by 24 hours of reperfusion because damage to the myocardium using this model does not result in transmural infarctions. Infarctions caused by 75 minutes of ischemia and reperfusion are characterized by patchy, endocardial, and mid MIs. The fact that almost the entire AAR was infarcted in 2 pigs suggests that reperfusion did not occur after releasing the coronary ligation. It is very likely that this is caused by thrombus formation, despite an intensive anticoagulant regime. Pigs are known for their hypercoagulability.31 For this reason, we used acetylsalicylic acid, clopidogrel (both loaded before surgery), and heparin before ischemia to target 3 different pathways involved in coagulation: Tromboxane-$A_2$ and ADP receptor–mediated platelet aggregation, and Thrombin/FXa-mediated coagulation, respectively. Nevertheless, the individual coagulability in these 2 outbred pigs may have varied.

Figure 4. OPN-305 improves systolic performance after ischemia/reperfusion injury. Baseline and postinfarct (A) global ejection fractions (EF) are shown. B, Fractional shortening (FS); *$P = 0.013$ compared with saline and †$P = 0.023$ compared with 12.5 mg/kg treatment. C, Systolic wall thickening (SWT) of the infarcted myocardium; *$P = 0.006$ and **$P = 0.021$ compared with saline and †$P = 0.003$ compared with 12.5 mg/kg treatment. D, Wall thickness (WT) of the infarcted myocardium. Each bar represents mean±SEM.
and thus may have caused thrombus formation despite 3 different anticoagulants.

Finally, OPN-305 significantly improved systolic performance of the ischemic/reperfused myocardium. The systolic wall thickening (the percentage increase of myocardial thickening during contraction) within the affected myocardium was significantly higher in OPN-305–treated pigs compared with saline treatment, in a dose-dependent manner. Moreover, saline and 1.56 mg/kg OPN-305 treatment resulted in a complete loss of contractile performance (bulging; as shown by a negative SWT index), whereas animals treated with 12.5 and 6.125 mg/kg OPN-305 still showed some residual contractility within the infarct area. These findings are in line with the higher myocardial viability seen in OPN-305–treated animals, as shown by the TTC staining and assessed by serum Troponin I levels. FS (measured by the percentage change in left ventricular diameter perpendicular to the infarct and remote region) was also significantly improved in OPN-305–treated animals. The fact that saline treatment resulted in similar global EF probably is attributable to compensatory actions of adjacent nonischemic regions in these animals. In the current study, only the center of the AAR and the remote myocardium (septal wall) were used to assess local function.

Limitations
We were not able to show reduced inflammation in the present study or to provide some mechanistic data by which OPN-305 exerts its therapeutic effect. Although there was a trend toward reduced WBC in porcine peripheral blood, a clear reduction in leukocyte infiltration in the ischemic/reperfused heart is lacking. We have previously demonstrated that leukocyte activation after I/R injury is reduced on TLR2 inhibition. Another mechanism, and possibly even a phase before leukocyte activation, may be reduced platelet activation. It has been shown that TLR2 expression on platelets is inhibition. Another mechanism, and possibly even a phase before leukocyte activation, may be reduced platelet activation. It has been shown that TLR2 expression on platelets is

In our opinion, there are 3 factors responsible for the lack of mechanistic evidence. First, it is well known that porcine material is difficult to use for biochemical and/or histological assessments because of the lack of specific antibodies and assays. Still, some groups do report molecular and histological assessment in porcine material. We tried 2 different manufacturers but failed to stain specific porcine neutrophils in our paraffin-embedded tissues. Finally, our open-chest model is already a strong inflammatory trigger as a result of the surgery, thereby overshadowing infarct-induced innate immune responses.

A second limitation is the fact that our follow-up period was 24 hours. A longer follow-up period is desirable to observe the effects on cardiac remodeling and performance in the long term.

In conclusion, administration of OPN-305 15 minutes before reperfusion results in a significant reduction of IS and improves systolic performance in a porcine I/R model. The efficacy in this large-animal model indicates that it has great clinical potential. Currently, OPN-305 is being tested in phase I clinical studies. Our study provides a solid rationale to assess the therapeutic efficacy of OPN-305 in patients with acute MI.

Acknowledgments
We thank the following persons for their biotechnical assistance: Cees Verlaan, Marlijn Jansen, Joyce Visser, Marina Emons, Merel Schurink, Hester de Bruin, and Evelyn Velema.

Sources of Funding
This work was supported by research grants from The Netherlands Organization for Scientific Research (NWO) and Utrecht University Mozalek grant (contract 017.004.004 to Dr Arslan), and The Netherlands Heart Foundation (contract 2010.T001 to Dr Arslan).

Disclosures
Dr O’Neill is cofounder and shareholder of Opsona Therapeutics Ltd. Dr Keogh, Mary Reilly, Dr McGuirk, and Dr McCormack are employees of Opsona Therapeutics Ltd.

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Circ Cardiovasc Interv. 2012;5:279-287; originally published online February 21, 2012; doi: 10.1161/CIRCINTERVENTIONS.111.967596
Circulation: Cardiovascular Interventions is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1941-7640. Online ISSN: 1941-7632

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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